

Peptide-Based Gemini Compounds

This invention relates to newly identified peptide-based gemini surfactant compounds, to the use of such compounds and to their production. The invention 5 also relates to the use of the peptide-based gemini compounds to facilitate the transfer of compounds into cells for drug delivery.

Surfactants are substances that markedly affect the surface properties of a liquid, even at low concentrations. For example surfactants will significantly reduce surface tension when dissolved in water or aqueous solutions and will reduce interfacial 10 tension between two liquids or a liquid and a solid. This property of surfactant molecules has been widely exploited in industry, particularly in the detergent and oil industries. In the 1970s a new class of surfactant molecule was reported, characterised by two hydrophobic chains with polar heads which are linked by a hydrophobic bridge (Deinega,Y *et al.*, *Kolloidn. Zh.* **36**, 649, 1974). These molecules, which have been 15 termed "gemini" (Menger, FM and Littau,CA, *J.Am.Chem.Soc.* **113**, 1451, 1991), have very desirable properties over their monomeric equivalents. For example they are highly effective in reducing interfacial tension between oil and water based liquids and have a very low critical micelle concentration.

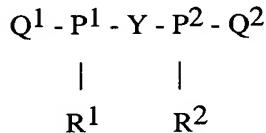
Cationic surfactants have been used *inter alia* for the transfection of 20 polynucleotides into cells in culture, and there are examples of such agents available commercially to scientists involved in genetic technologies (for example the reagent TfxTM-50 for the transfection of eukaryotic cells available from Promega Corp. WI, USA).

The efficient delivery of DNA to cells *in vivo*, either for gene therapy or for 25 antisense therapy, has been a major goal for some years. Much attention has concentrated on the use of viruses as delivery vehicles, for example adenoviruses for epithelial cells in the respiratory tract with a view to corrective gene therapy for cystic fibrosis (CF). However, despite some evidence of successful gene transfer in CF patients, the adenovirus route remains problematic due to inflammatory side-effects 30 and limited transient expression of the transferred gene. Several alternative methods for *in vivo* gene delivery have been investigated, including studies using cationic surfactants. Gao,X *et al.* (1995) *Gene Ther.* **2**, 710-722 demonstrated the feasibility of this approach with a normal human gene for CF transmembrane conductance regulator (CFTR) into the respiratory epithelium of CF mice using amine carrying cationic lipids.

This group followed up with a liposomal CF gene therapy trial which, although only partially successful, demonstrated the potential for this approach in humans (Caplen, NJ. *et al.*, *Nature Medicine*, 1, 39-46, 1995). More recently other groups have investigated the potential of other cationic lipids for gene delivery, for example 5 cholesterol derivatives (Oudrhiri,N *et al.* *Proc.Natl.Acad.Sci.* **94**, 1651-1656, 1997). This limited study demonstrated the ability of these cholesterol based compounds to facilitate the transfer of genes into epithelial cells both *in vitro* and *in vivo*, thereby lending support to the validity of this general approach.

These studies, and others, show that in this new field of research there is a 10 continuing need to develop novel low-toxicity surfactant molecules to facilitate the effective transfer of polynucleotides into cells both *in vitro* for transfection in cell-based experimentation and *in vivo* for gene therapy and antisense treatments. The present invention seeks to overcome the difficulties exhibited by existing compounds.

The invention relates to the peptide-based gemini compounds comprising two 15 linked chains:



each chain having:

20 (1) a positively charged hydrophilic head, Q¹ or Q², formed from one or more amino acids and/or amines;
 (2) a central portion, P¹ or P², having a polypeptide backbone; and
 (3) a hydrophobic tail, R¹ or R²;
 the central sections of each chain being linked together by bridge Y through residues in 25 P¹ and P².

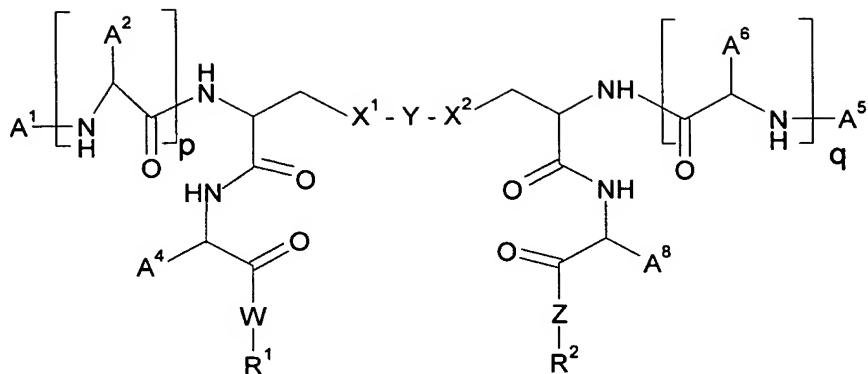
Preferably the central portion is made up of two or three amino acids, P^a (optional), P^b and P^c, in which:

P^a is a D- or L- amino acid, preferably hydrophilic, such as threonine or serine,

P^b is preferably D- or L- cysteine, serine or threonine, and

30 P^c is preferably D- or L- serine or threonine and is linked to R¹ or R².

Preferred compounds of the present invention include compounds of the formula (I):



(I)

5

where:

A^1 and A^5 , which may be the same or different, is a positively charged group formed from one or more amino acids or amines joined together in a linear or branched manner and preferably bonded by an amide (CONH) bond;

10 A^2/A^6 CH(NH)CO, which may be the same or different, is derived from an amino acid, preferably serine;

p and q , which may be the same or different, is 0 or 1;

X^1/X^2 CH₂CH(NH)CO, which may be the same or different, is derived from cysteine (X^1/X^2 = S), serine or threonine (X^1/X^2 = O);

15 A^4/A^8 CH(NH)CO, which may be the same or different, is derived from serine or threonine;

Y is a linker group, preferably $(CH_2)_m$ where m is an integer from 1 to 6, most preferably 2, and may be a disulphide bond when X^1 and X^2 is each S;

R^1 and R^2 are $C_{(10-20)}$ saturated or unsaturated alkyl groups, and

20 W and Z are NH, O, CH₂ or S; or

a salt, preferably a pharmaceutically acceptable salt thereof.

Preferably, the compound is symmetrical, that is A^1 and A^5 are the same, A^2 and A^6 are the same, A^4 and A^8 are the same, R^1 and R^2 are the same, and W and Z are the same.

25 Representative examples of A¹/A⁵ include D- or L-amino acids selected from arginine, lysine, ornithine and histidine, preferably lysine, or amines such as spermine and spermidine. Up to seven amino acids and /or amines may be linked in a linear or

branched chain. Preferred examples include groups having two or three lysines or ornithines or a combination of lysine, ornithine, arginine and histidine, for instance:



or



or



in which R is H or $\text{NHCO}(\text{NH}_2)(\text{CH}_2)_4\text{NH}_2$ or $\text{NHCO}(\text{NH}_2)(\text{CH}_2)_3\text{NH}_2$

Preferably, $-\text{X}^1\text{-Y-X}^2-$ is $-\text{SCH}_2\text{CH}_2\text{S}-$ or $-\text{OCH}_2\text{CH}_2\text{O}-$

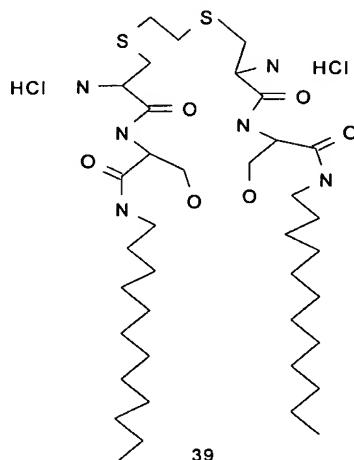
10 Preferably, R^1 and R^2 is each a $\text{C}_{12}\text{-C}_{20}$ alkyl group, for instance C_{12} .

Preferably, W and Z is NH, thereby forming a further amide (CONH) bond.

Compounds of the present invention may be prepared from readily available starting materials using synthetic peptide chemistry well known to the skilled person.

For preferred compounds of the present invention a useful intermediate is the

15 compound:



which is synthesised in a multi-stage process beginning, for instance, with the

20 construction of the di-cysteine part and subsequently building up the hydrophilic head by attaching a serine moiety at the carboxyl group of each cysteine moiety, using standard peptide chemistry, and then attaching the hydrocarbon chains to the carboxyl group of the serine moiety using a standard amide forming reaction well known to those skilled in the art. This intermediate can then be taken through to compounds of 25 formula (I) by further reaction at the nitrogens of the cysteine residues.

Another aspect of the invention relates to methods for using the peptide-based gemini compounds. Such uses include facilitating the transfer of oligonucleotides and polynucleotides into cells for antisense, gene therapy and genetic immunisation (for the generation of antibodies) in whole organisms. Other uses include employing the 5 compounds of the invention to facilitate the transfection of polynucleotides into cells in culture when such transfer is required, in, for example, gene expression studies and antisense control experiments among others. The polynucleotides can be mixed with the compounds, added to the cells and incubated to allow polynucleotide uptake. After further incubation the cells can be assayed for the phenotypic trait afforded by the 10 transfected DNA, or the levels of mRNA expressed from said DNA can be determined by Northern blotting or by using PCR-based quantitation methods for example the Taqman® method (Perkin Elmer, Connecticut, USA). Compounds of the invention offer a significant improvement, typically between 3 and 6 fold, in the efficiency of cellular uptake of DNA in cells in culture, compared with compounds in the previous 15 art. In the transfection protocol, the gemini compound may be used in combination with one or more supplements to increase the efficiency of transfection. Such supplements may be selected from, for example:

(i) a neutral carrier, for example dioleyl phosphatidylethanolamine (DOPE) (Farhood, H., *et al* (1985) *Biochim. Biophys. Acta* 1235 289);

20 (ii) a complexing reagent, for example the commercially available PLUS reagent (Life Technologies Inc. Maryland, USA) or peptides, such as polylysine or polyornithine peptides or peptides comprising primarily, but not exclusively, basic amino acids such as lysine, ornithine and/or arginine. The list above is not intended to be exhaustive and other supplements that increase the efficiency of transfection 25 are taken to fall within the scope of the invention.

In still another aspect, the invention relates to the transfer of genetic material in gene therapy using the compounds of the invention.

Yet another aspect of the invention relates to methods to effect the delivery of non-nucleotide based drug compounds into cells *in vitro* and *in vivo* using the 30 compounds of the invention.

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"Amino acid" refers to dipolar ions (zwitterions) of the form $^+H_3NCH(R)CO_2^-$. They are differentiated by the nature of the group R, and when R

is different from hydrogen can also be asymmetric, forming D and L families. There are 20 naturally occurring amino acids where the R group can be, for example, non-polar (e.g. alanine, leucine, phenylalanine) or polar (e.g. glutamic acid, histidine, arginine and lysine). In the case of un-natural amino acids R can be any other group 5 which is not found in the amino acids found in nature.

“Polynucleotide” generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. “Polynucleotides” include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and 10 double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, “polynucleotide” refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or 15 RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. “Modified” bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications have been made to DNA and RNA; thus, “polynucleotide” embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found 20 in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. “Polynucleotide” also embraces relatively short polynucleotides, often referred to as oligonucleotides.

“Transfection” refers to the introduction of polynucleotides into cells in culture using methods involving the modification of the cell membrane either by 25 chemical or physical means. Such methods are described in, for example, Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). The polynucleotides may be linear or circular, single-stranded or double-stranded and may include elements controlling replication of the polynucleotide or expression of homologous or 30 heterologous genes which may comprise part of the polynucleotide.

The invention will now be described by way of the following descriptions and examples.

DESCRIPTIONS

Description 1. *Bisthioether 3*

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A 1L 3-necked flask equipped with mechanical stirrer, reflux condenser and dropping funnel was flushed with N₂ directly into the flask through the condenser. A solution of 31.3g (0.20mole) L-cysteine.hydrochloride.xH₂O (**1**) in 100ml (degassed ultrasonic for 10 minutes) was added to the flask. A degassed solution of 10 34g (0.40mole) NaHCO₃ in 300ml H₂O was added, followed by the dropwise addition (30 minutes) of a degassed solution of 18.8g (8.6ml; 0.10mole) 1,2-dibromoethane (**2**) in 100ml EtOH. After another 30 minutes the mixture was heated to 65-70°C and stirred, still under N₂, for another 3 hours (within 1 minutes precipitation started). The mixture was cooled to 20°C, filtered, rinsed with 30ml H₂O and with 100ml acetone (2x). After drying 19.4g white solid was obtained which still contained some free cysteine (¹H NMR). The solid was suspended in 15 250ml 2.5%NH₄OH and 25%NH₄OH was added until a clear solution was obtained. To this solution 15mg KCN was added and the mixture was stirred for 30 minutes. The solution was acidified to pH6 using HOAc and stirred for 30 minutes whilst 20 cooling to 5°C. The solid was collected, rinsed with H₂O (100ml), acetone (2 x 100ml) and dried, yielding 18.1g (68%) **3** as a white solid.

Description 2. *Boc-L-Leucine 5*

25 19.7g (0.15mole) L-Leucine **4** was suspended in 200mlH₂O and 6.75g (0.17mole) NaOH was added. The clear solution was cooled to <10°C and a solution of 36g (0.165mole) (BOC)₂O in 100ml THF was added dropwise keeping T < 10°C (30 minutes). After stirring for 4 hours at room temperature the mixture was acidified to pH2 by adding 1N HC1. The mixture was extracted with EtOAc (250, 100 and 30 100ml), the combined organic layers were dried on Na₂SO₄ and evaporated, yielding 40g (>100%) **5** as a colorless oil which contained some THF but was used as such.

Description 3. *Boc-L-Leucine-OSuc 7*

40g crude 5 (max. 0.15mole) was dissolved in 400ml THF (distilled prior to use from LAH) under N₂. After addition of 17.3g (0.15 mole) N-hydroxysuccinimide (6) and 30.9g (0.15mole) DCC the mixture was stirred for 3.5 hours at room temperature. The mixture was filtered over a P₂ glassfilter, the filter was rinsed with 50ml THF
5 and the filtrate was evaporated. The residue was dissolved in 400ml refluxing isopropylether, the solution was filtered hot and the filtrate was placed at 4°C for 20 hours. The solid was collected, rinsed with 50ml IPE and dried, yielding 32g (65%) 7 as a white solid.

10 **Description 4. Compound 8**

8.05g (30mmole) 3 was suspended in 200ml H₂O and after addition of 8.3g (60mmole) K₂CO₃ the mixture was heated to get a clear solution. After cooling to room temperature a solution of 19.7g (60mmole) 7 in 200ml THF was added at once.
15 The mixture was stirred at room temperature for 20 hours, followed by acidification to pH 6 (30% HC1). After filtration the filtrate was acidified to pH2-3 (30% HC1) and extracted with CHC1₃ (250, 100 and 100ml). The combined organic layers were once washed with brine (50ml), dried on Na₂SO₄ and evaporated, yielding 23g crude 8 as a white foam which was used as such.

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Description 5. Compound 9

23g crude 8 was dissolved in 200ml EtOAc, cooled to 0°C and HC1-gas was bubbled through for 1 hour, followed by stirring at this temperature for another hour. The
25 solid was collected, rinsed with ether and dried under vacuo over KOH. This yielded 15.4g (91%) 9 as a hygroscopic nearly white solid.

Description 6. Compound 10

30 17.0g (30mmole) 9 dissolved in 250ml H₂O and after cooling to < 10°C in an ice/waterbath 7.2g (180mmole) NaOH was added. After stirring for 10 minutes at room temperature a solution of 14.4g (66mmole) lauroylchloride in 50ml THF was added dropwise in 5 minutes. The mixture was stirred for 20 hours and extracted with hexane (2 x 150 ml). A 3-layer system formed and the lower 2 layers were

acidified to pH 1-2 (1 N HC1) and extracted with ether (200, 100, 100 and 50ml). The combined ether layers were dried on MgSO₄ and evaporated, yielding 24g (93%) **10** as an oil/foam. This material was used as such.

5 **Description 7. Compound 11**

24g (max 27.5mmole) **10** dissolved in 400ml THF (distilled from LAH) under N₂. After addition of 6.33g (55mmole) N-hydroxysuccinimide (**6**) and 11.33g (55mmole) DCC the mixture was stirred at room temperature for 20 hours. The mixture was 10 filtered over a large P₂ glassfilter, another 250ml THF was added to speed up filtration. The filter was rinsed with another 100ml THF. The filtrate was evaporated yielding 40g white solid and this crude material was recrystallized from 400ml IPA. Stirred 1 hour 0°C and collected. After drying 22.3g (77%) **11** was obtained as white solid.

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Description 8. Compound 13

542mg (2.2mmole) H.Arg.NH₂.2HC1 (**12**) was dissolved in 15ml H₂O and 304mg (2.2mmole) K₂CO₃ was added. A solution of 1.05g (1.0mmole) **11** in 15ml THF 20 was added at once and the mixture was stirred at room temperature for 20 hours. Most of the THF was evaporated and an oil formed in the waterlayer. This suspension was extracted with ether (6 x 50 ml), the combined ether layers were dried on MgSO₄ and evaporated, yielding 9500 mg (82%) **13 free amine** as a yellow solid. Treatment of this material with HC1-gas in EtOAc/CH₂C1₂ gave **13** as a 25 yellow solid.

Description 9. Compound 15

5.25g (5.0mmole) **11** dissolved in 50ml THF (some heating was needed) and a 30 solution of 1.31g (10.5mmole) Taurine (**14**) and 1.45 g (10.5mmole) K₂CO₃ in 50ml H₂O was added at once. After stirring at room temperature for 20 hours most of the THF was removed by evaporation and 300ml MeOH was added. Mixture placed at -20°C for 20 hours, solid collected, rinsed with MeOH and dried. This yielded 3.3g **15** contaminated with N-hydroxysuccinimide., which was combined with 700mg

impure material of an other run. This 4.0g was recrystallized from 200ml MeOH + 50ml H₂O. Some solid was removed by filtration and the clear filtrate was placed at -20°C for 2 hours, the solid was colected, rinsed with MeOH and dried. This yielded 2.0g 15 as an off white solid. A 2nd crop of 800mg was obtained from the filtrate.

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Description 10. *Compound 16*

1.05g (1.0mmole) 11 was dissolved in 20ml THF and a solution of 275mg (2.2mmole) 2-aminoethylphosphonic acid and 300mg (2.2mmole) K₂CO₃ in 20ml H₂O was added at once. After stirring at room temperature for 20 hours most of the THF was removed by evaporation and the aqueous solution was freeze dried. This yielded a white solit which was recrystallized from 20ml MeOH and placed at -20°C for 20 hours. The solid was collected and dried yielding 150mg. ¹H NMR showed it to be N-hydroxysuccinimide. The filtrate was evaporated and the remaining yellow oil was dissolved in 10ml refluxing MeOH and after addition of 20ml IPA placed at -20°C for 4 hours. Solid was collected and dried, yielding 260mg. The filtrate was evaporated, dissolved in 20ml EtOAc and placed at -20°C for 20 hours. The solid was collected.

20 **Description 11. *Compound 17***

1.05g (1.0mmole) 11 was dissolved in 20ml THF and a solution of 310mg (2.2mmole) O-phosphocolamine and 300mg (2.2mmole) K₂CO₂ in 20ml H₂O was added at once. After stirring at room temperature for 20 hours most of the THF was removed by evaporation and the aqueous solution was freeze dried. The white solid was recrystallized from 20ml MeOH and placed at -20°C for 20 hours. The solid was collected, rinsed with MeOH and dried. This yielded 310mg white solid. No product.

The filtrate was evaporated and the remaining yellow solid was dissolved in 10ml MeOH, 20ml IPA was added and the mixture was placed at -20°C for 20 hours.

Solid collected, 20-30mg

Filtrate evaporated, residue dissolved in 25ml EtOAc, placed at -20°C and after 20 hours the solid was collected.

Description 12. *Boc-Glycine Boc-Glycine* 19

18.8g (0.25mole) Glycine 18 was suspended in 250ml H₂O and 11g (0.275mole) NaOH was added. The clear solution was cooled to <10°C and a solution of 60g (0.165mole) (BOC)₂O in 250ml THF was added dropwise keeping T < 10°C (20 minutes). After stirring for 20 hours at room temperature the mixture was acidified to pH1 by adding 1N HC1. The mixture was extracted with EtOAc (250, 100 and 100ml), the combined organic layers were dried on MgSO₄ and evaporated, yielding 47.5g (>100%) 5 as a colourless oil which contained some THF but was used as such.

Description 13. *Boc-Glycine-OSuc* 20

47.5g crude 19 (max. 0.25mole) was dissolved in 500ml THF (distilled prior to use from LAH) under N₂. After addition of 30g (0.26mole) N-hydroxysuccinimide (6) and 53.5g (0.26mole) DCC at < 10°C, the mixture was stirred for 20 hours at room temperature. Now the mixture was filtered over 1cm celite on a P2 glassfilter, the filter was rinsed with 200ml THF and the filtrate was evaporated. The crude material (56g) was recrystallized from refluxing isopropylether/THF (600ml 1:1), the solution was filtered hot and the filtrate was stirred at 0°C for 3 hours. The solid was collected, rinsed with 50ml IPE and dried, yielding 16.4g (24%) 20 as a white solid. Filtrate evaporated and stored.

Description 14. *Compound* 21

8.05g (30mmole) 3 was suspended in 200ml H₂O and after addtion of 8.3g (60mmole) K₂CO₃ the mixture was heated to get a clear solution. After cooling to < 40°C a solution of 16.3g (60mmole) 20 in 200ml THF was added in 4 portions within 2 minutes. The mixture was stirred at room temperature for 72 hours, followed by acidification to pH 6 (30% HC1). After filtration the filtrate was acidified to pH 2-3 (30% HC1) and extracted with CHCl₃ (250, 100 and 100ml). The combined organic layers were once washed with brine (50ml), dried on MgSO₄ and evaporated, yielding 15.8g (90%) 21 as a white foam which was used as such.

Description 15. Compound 22

15.8g (27mmole) crude 21 was dissolved in 300ml EtOAc and HCl-gas was bubbled through for 1 hour, followed by stirring at 0°C in an ice/waterbath and a solution of 5 12.2g (56mmole) lauroylchloride in 50ml THF was added at once. The mixture was stirred for 20 hours and extracted with hexane (2 x 100ml). The water layer was acidified to pH 1-2 (1 N HCl) and extracted with ether (3 x 150ml). A solid formed during extraction, which was collected and dried. This yielded 7.5g (39%) 23 white solid.

10 The filtrate was evaporated and the remaining slurry was stirred in 250ml Et₂O. An attempt to collect the solid failed and addition of 25ml MeOH gave a clear solution. This solution was placed at -20°C for 20 hours. The solid was collected, rinsed with ether and dried.

15 Description 16. Compound 24

7.47g (10mmole) 23 dissolved in 200ml THF (distilled from LAH) under N₂. After addition of 2.53g (22mmole) N-hydroxysuccinimide (6) and 4.53g (22mmole) DCC the mixture was stirred at room temperature for 72 hours. The mixture was filtered 20 over a 1cm layer celite on a large P₂ glassfilter (very slow). The filtrate was evaporated yielding 1.90g 24 as a foam.

The filer was rinsed with 300ml dioxane and the filtrate was evaporated yielding 6.9g 24 as a foam. Total yield 8.8g (94%).

25 Description 17. Compound 25

542mg (2.2mmole) H.Arg.NH₂.2HCl (12) was dissolved in 15ml H₂O and 750mg (5.4mmole) K₂CO₃ was added. A solution of 941mg (1.0mmole) 24 in 15ml THF was added at once and the mixtrue was stirred at room temperature for 20 hours.

30 Most of the THF was evaporated and the waterlayer was extracted with EtOAc (2 x 50 ml), the combined EtOAc layers were dried on MgSO₄ and evaporated, yielding 150mg 25 free amine as a foam. Both portions were combined and dissolved in CH₂C₁₂ and after the addition of 75ml EtOAc, HCl-gas was bubbled through for 1.5 hour. Now the mixture was cooled to 0°C and stirred for another 2 hours.

Attempts to collect the solid failed, so 100ml ether was added and the mixture was stirred at room temperature for 20 hours. The solid was collected, rinsed with ether and dried, yielding 520mg 25 as a slightly brown solid.

5 **Description 18. Compound 26**

250mg (2.0mmole) taurine (14) was dissolved in 15ml H₂O and 280mg (2.0mmole) K₂CO₃ was added. Now a solution of 941mg (1.0mmole) 24 in 15ml THF was added at once and the mixture was stirred at room temperature for 20 hours. Most of the THF was evaporated and the aqueous solution was freeze dried. The resulting white solid was recrystallized from 30ml MeOH, stirred 3 hours at 0°C and the solid was collected, rinsed with ether and dried.

This yielded 225mg 26 as a white solid.

10 The filtrate was partly evaporated and placed at -20°C for 20 hours. The solid was collected, rinsed with ether and dried, yielding 210mg 26 as a white solid. Both portions were combined.

Description 19. Compound 27

20 26.8g (0.1 mole) 3 was suspended in 300ml H₂O and 9.6g (0.24mole) NaOH was added. A clear solution formed within 5 minutes, the mixture was cooled to < 10°C and a solution of 43.6g (0.2mole) BOC₂O in 300ml THF was added dropwise in 30 minutes. The mixture was stirred at room temperature overnight. After addition of a solution of 2.5g (0.06mole) NaOH in 25ml H₂O and 15g (0.07 mole) BOC₂O in

25 75ml THF the mixture was stirred for another 18 hours.

The mixture was acidified to pH 2 by adding 2N HCl and after addition of 300ml brine, extracted with THF (3 x 400ml) and EtOAc (2 x 300ml) The combined organic layers were dried on MgSO₄ and evaporated, yielding 42g white solid. This solid was recrystallized from MEK/pentane, stirred at room temperature for 2 hours and placed at -20°C for 2 hours. The solid was collected and dried, yielding 37.8g (81%) 27 as a white solid.

Description 20. Compound 28

8.9g (19mmole) 27 was dissolved in 300ml THF (from LAH) under N₂ and 4.37g (38mmole) 6 and 7.38g (38mmole) DCC were added. After stirring at room temperature for 18 hours the mixture was filtered over 1cm celite, the filter was rinsed with another 300ml THF and the filtrate was evaporated yielding 11.1g (88%) 28 as a white solid.

Description 21. Compound 29

2.7g (20.6mmole) L-leusine (4) and 2.8g (20.3mmole) K₂CO₂ were dissolved in 100ml H₂O and a suspension of 6.6g (10mmole) 28 in 50ml dioxane was added. The mixture was stirred at room temperature for 20 hours and most of the dioane was removed by evaporation. The aqueous solution was extracted with 50ml ether and acidified to pH 1 by addition of 30% HC1. Now the mixture was extracted with CHCl₃ (150, 100 and 50ml), the combined organic layers were washed with brine (200ml), dried on MsSO₄ and evaporated, yielding a white foam which was stripped with THF to get 7.5g (>100%) 29 as a solid white foam.

Description 22. Compound 30

20 7.5g crude 29 (max. 10 mmole) was dissolved in 100 ml THF (from LAH) under N₂, 2.3g (20mmole) N-hydroxysuccinimide (6) and 4.12g (20mmole) DCC were added and the mixture was stirred at room temperature for 20 hours. The mixture was filtered over 1cm celite, the filter was rinsed with THF and the filtrate was evaporated, yielding 9.5g white foam which was recrystallized from 75ml IPA and 25 placed at -20°C for 3 hours. The solid was collected but liquified immediately on the glass filter. The oily material was dissolved in 20ml THF and evaporated, yielding 6.5g (73%) 30 as a solid white foam.

Description 23. Compound 31

30 6.5g (7.3mmole) 30 was dissolved in 100ml THF and after addition of 2.78g (15mmole) dodecylamine the mixture was stirred at room temperature for 18 hours. After evaporation a foam was obtained which was dissolved in 100ml CHCl₃. The

solution was washed with H₂O (2 x 75 ml), dried on MgSO₄ and evaporated, yielding 7.5g (100%) 31 as a solid foam.

Description 24. Compound 32

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7.5g (7.3mmole) crude 31 was dissolved in 250ml EtOAc under heating and after cooling to room temperature HCl-gas was bubbled through for 2 hours. Stirring was continued at 0°C for 3 hours. The solid was collected, rinsed with ether and dried under vacuo over KOH. This yielded 4.0g (60%) 32 as a white solid.

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Description 25. Compound 33

903mg (1.0mmole) 32 was dissolved in 10ml H₂O under heating (gel formed), after cooling to room temperature, 80mg (2.0mmole) NaOH dissolved in 2ml H₂O was added. A suspension was formed and THF was added until a clear solution was obtained. Now a solution of 572mg (2mmole) BOC-β-alalOSuc (42) in 5ml THF was added and the mixture was stirred at room temperature for 5 hours. Most of the THF was removed by evaporation, another 30ml H₂O was added and after stirring for another hour the solid was collected, rinsed with 10 ml H₂O and dried. This yielded 1.0g (85%) 33 as an off white solid.

Description 26. Compound 34

1.0g (0.85mmole) 33 suspended in 25ml EtOAc and 25ml CH₂Cl₂ added to get a clear solution. HCl-gas bubbled through for 1.5 hour and stirred at 0°C for another 2 hours. No solid had formed so most of the CH₂Cl₂ was removed by evaporation and stirring at 0°C was continued for another 30 minutes. The solid was collected, partly by filtration (very slow), mainly by centrifugation. Total yield after drying 810mg (91%) 34 as a yellow solid.

30

Description 27. Compound 36

4.2g (40mmole) L-serine 35 and 5.53 (40mmole) K₂CO₃ were dissolved in 300ml H₂O and a suspension of 12.8g (max. 19mmole) 28 in 300ml THF was added. The

mixture was stirred at room temperature for 72 hours and most of the THF was removed by evaporation. The aqueous solution was acidified to pH 1 by addition of 1N HCl. The mixture was extracted with $\text{CH}_2\text{Cl}_2 + 15\%$ MeOH (250, 100 and 100ml), the combined organic layers were dried on MgSO_4 and evaporated, yielding 8.5g (70%) 36 as a white solid foam which was used as such.

Description 28. Compound 37

8.5g (max. 13.2mmole) 36 was dissolved in 200ml THF (from LAH) under N_2 and after addition of 3.46g (30mmole) N-hydroxysuccinimide (6) and 6.2g (30mmole) DCC the mixture was stirred at room temperature for 24 hours. The mixture was filtered over 1cm celite, the filter was rinsed with 50ml THF and evaporated. This yielded 12.5g (>100%) 37 as a white foam which was used as such.

15 Description 29. Compound 38

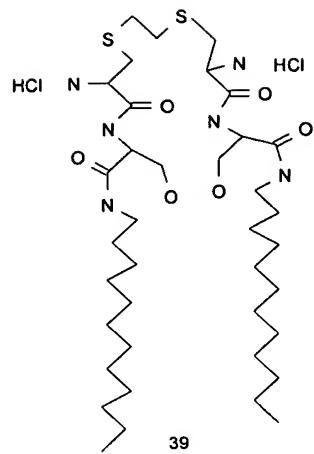
12.5g crude (max 13.2mmole) 37 was dissolved in 200ml THF and stirred with 5.0g (27mmole) dodecylamine at room temperature for 48 hours. The THF was removed by evaporation and the residue was dissolved in 250ml CHCl_3 and extracted with brine (2 x 150ml). The combined brine layers were extracted with 50ml CHCl_3 and the combined CHCl_3 layers were dried on MgSO_4 and evaporated. This yielded 15.4g (>100%) 38 as a nearly white solid which was used as such.

25

EXAMPLES

Example 1. Compound 39 2-amino-3-{2-[2-amino-2-(1-dodecylcarbamoyl-2-hydroxy-ethylcarbamoyl)-ethylsulphonyl]-ethylsulphonyl}-N-(1-dodecylcarbamoyl-2-hydroxy-ethyl)-propionamide

15.4g (max. 13.2mmole) 38 dissolved in 400ml EtOAc and HCl-gas was bubbled through for 1.5 hour. The mixture was stirred at 0°C for 2 hours, the solid was collected, rinsed with ether and dried, yielding 9.9g (88%) 39 as a white solid.



5

Example 2. Compound 40

4.25g (5mmole) **39** was dissolved in 100ml H₂O with heating and after cooling to <40°C a solution of 460mg (10mmole) NaOH in 10ml H₂O was added. A suspension formed and THF was added until a clear solution was obtained (150ml). Next 2.86g (10mmole) BOC-β-alaOSu (**42**) was added and the mixture was stirred at room temperature for 20 hours. Most of THF was removed by evaporation, another 100ml H₂O was added and the mixture was stirred at 0°C for 3 hours. The solid was collected, rinsed with 20ml H₂O and dried. This yielded 5.2g (93%) **40** as a nearly white solid.

Example 3. Compound 41

5.2g (4.6mmole) **40** was dissolved in 100ml CH_2Cl_2 and 200ml EtOAc was added.
20 HCl-gas was bubbled through for 1.5 hour and stirring was continued at 0°C for 2 hours. The solid was collected, rinsed with ether and dried, yielding 4.7g (100%) **41** as off white solid.

Example 4. Compounds 42 and 43

After neutralization of compounds 34 and 41 using 2 eq. of NaOH in MeOH both compounds were treated with $(\text{CH}_2\text{O})_n$ and NaCNBH_3 under N_2 for 18 hours. In both reactions complex mixtures were formed, probably due to alkylation on amide nitrogen as well.

5

Example 5. Compound 44

332mg (0.39mmole) 39 was dissolved in 15ml H_2O under heating and after cooling to $< 40^\circ\text{C}$ a solution of 33mg (0.83mmole) NaOH in 1ml H_2O was added. A white 10 suspension formed and THF was added until a clear solution was obtained (25ml). To this solution 499mg (0.78mmole) BOC-Arg(Z)₂-OSu (47) was added and the mixture was stirred at room temperature for 20 hours. Most of the THF was evaporated and another 15ml H_2O was added. After 2 hours stirring the solid was collected, rinsed with H_2O and dried, yielding 700mg (98%) 44 as a white solid.

15

Example 6. Compound 45

100mg (0.05mmole) 44 was dissolved in 20ml HOAc and 500mg 10% Pd on Carbon (0.5mmole Pd) was added. The mixture was stirred under H_2 (5 bar) for 48 hours. 20 The mixture was filtered over 1cm celite, the filter was rinsed with 10ml HOAc and the filtrate was evaporated. This yielded 100mg crude 45 as a green oil.

Example 7. Compound 46

25 100mg crude 45 (max 0.05mmole) was dissolved in 10ml CH_2Cl_2 and 10ml EtOAc was added. HCl -gas was bubbled through for 1 hour and the mixture was stirred 18 hours at room temperature. Most of the CH_2Cl_2 was removed by evaporation, 30ml ether was added and the mixture was stirred at 0°C for 1 hour. No crystalline material had formed so the mixture was evaporated, yielding 75mg crude 46 as a 30 yellow oil.

Example 8. Compound 49

850mg (1.0mmole) 39 was dissolved in 20ml H₂O and after 88mg (2.2mmole) NaOH was added a suspension formed. Now THF was added until a clear solution was obtained (30ml) and 974mg (2.2mmole) BOC₂LysOSuc (compound 48) was added. After stirring at room temperature for 20 hours most of the THF was
5 removed by evaporation, another 20ml H₂O was added and the mixtrue was stirred for 2 hours. The solid was collected, rinsed with H₂O and dried, yielding 1.35g (90%) 49 as a white solid.

Example 9. Compound 50

10 500mg 49 was dissolved in 25ml CH₂C1₂ and after addition of 25ml EtOAc HC1-gas was bubbled through for 1 hour, the mixture was stirred at 0°C for 1.5 hour. An attempt to collect the solid failed, 40ml ether was added and stirring was continued for 18 hours. The solid was collected, rinsed with ehter and dried, yielding 290mg
15 50 as a white solid.

Example 10. Compound 51

20 425mg (0.43mmole) 41 was dissolved in 20ml H₂O and 44mg (1.1mmole) NaOH was added. A suspension formed and THF was added until a clear solution was obtained (25ml). After addition of 487mg (1.1mmole) 48 the mixture was stirred at room temperature for 20 hours. Most of the THF was evaporated, another 20ml H₂O was added and after stirring for 1.5 hour the solid was collected, rinsed with H₂O and dried. This yielded 750mg 51 as a white solid.

25 **Example 11. Compound 52**

250mg 51 was dissolved in 30ml CH₂C1₂ and after addition of 30ml EtOAc, HC1-gas was bubbled through for 1 hour, the mixture was stirred at 0°C for 1.5 hour. The
30 solid was collected, rinsed with ether and dried, yielding 120mg 52 as a white salt.

Example 12. Compound 57 (SucOSerLysBOC₂)

4.43g (10mmole) BOC₂LysOSuc (**48**) was dissolved in 50ml THF and a solution of 1.16g (11mmole) L-serine and 1.52g (11mmole) K₂CO₃ in 50ml H₂O was added immediately. The mixture was stirred at room temperature for 72 hours. Most of the THF was removed by evaporation and the remaining slurry was acidified to pH2 by 5 the addition of 1M HCl and extracted with CHCl₂ (2 x 75ml). The combined organic layers were dried (Na₂SO₄) and evaporated, yielding **57** as a white solid foam which was used as such in example 43.

Example 13. Compound 54

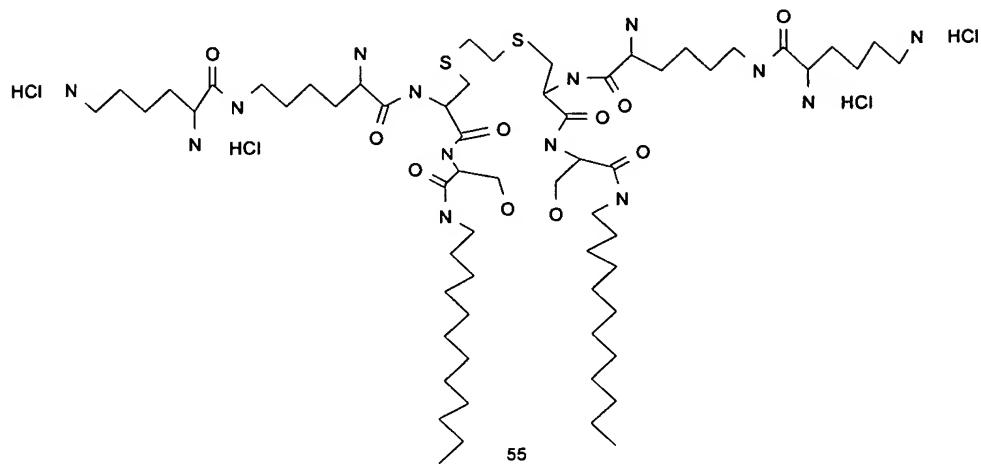
10 850mg (1.0mmole) **39** was dissolved in 30ml H₂O and 88mg (2.2mmole) NaOH was added, followed by the addition of 30ml THF to get a clear solution. A solution of 1.5g (max. 2.3mmole) **57** in 30ml THF was added immediately and the solution was stirred at room temperature for 48 hours. Most of the THF was removed by 15 evaporation, another 30ml H₂O was added and stirring was continued for 1 hour. Because no solid had formed, the mixture was extracted twice with 75ml EtOAc/ether (2:1). The combined organic layers were dried (Na₂SO₄) and evaporated yielding the BOC-protected intermediate as a solid foam.

20 This foam was dissolved in 25ml CH₂Cl₂ and 50ml EtOAc was added. HCl gas was bubbled through the clear solution for 1 hour and stirring was continued at 0°C for another hour. The salt was collected, rinsed with ether and dried under vacuum, yielding 1.15g (85%) **54** as a slightly brown solid.

25 **Example 14. Compound 55**

1.18g (1.0mmole) **50** was dissolved in 25ml H₂O and 176mg (4.4mmole) NaOH was added, followed by the addition of 30ml THF to get a clear solution. 974mg (2.2mmole) **48** were added and the mixture stirred at room temperature for 48 hours. Most of the THF was removed by evaporation, another 50ml H₂O was added and the 30 mixture was stirred for 2 hours. Because no solid was formed the mixture was extracted with ether (2 x 100ml), the combined organic layers were dried (Na₂SO₄) and evaporated, yielding 2g of the BOC-protected intermediate as a solid foam. The foam was dissolved in 50ml EtOAc and HCl-gas was bubbled through the solution for 1 hour and stirring was continued at 0°C for another hour. The salt was

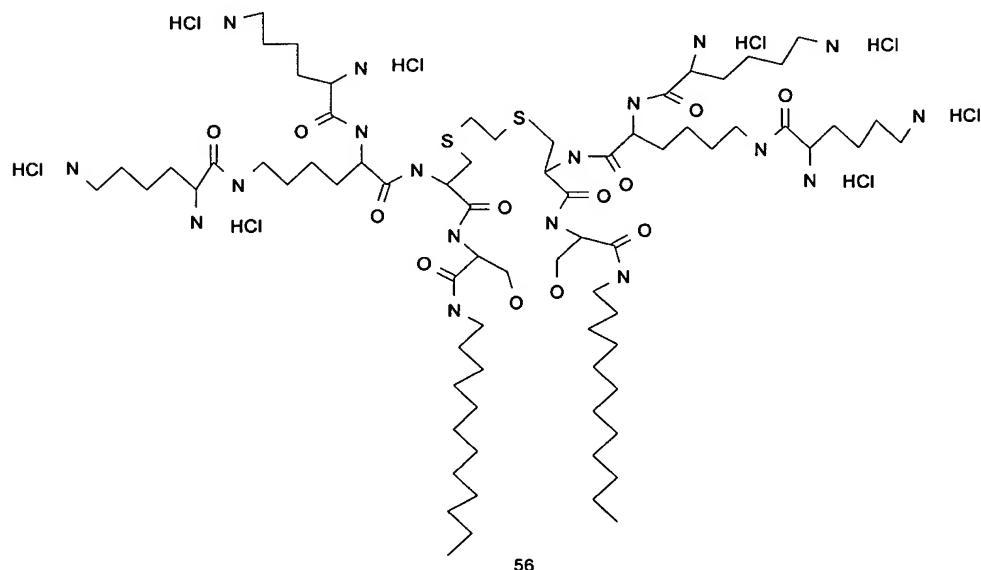
collected, rinsed with ether and dried under vacuum, yielding 1.15g (76%) 55 as a nearly white solid.



5

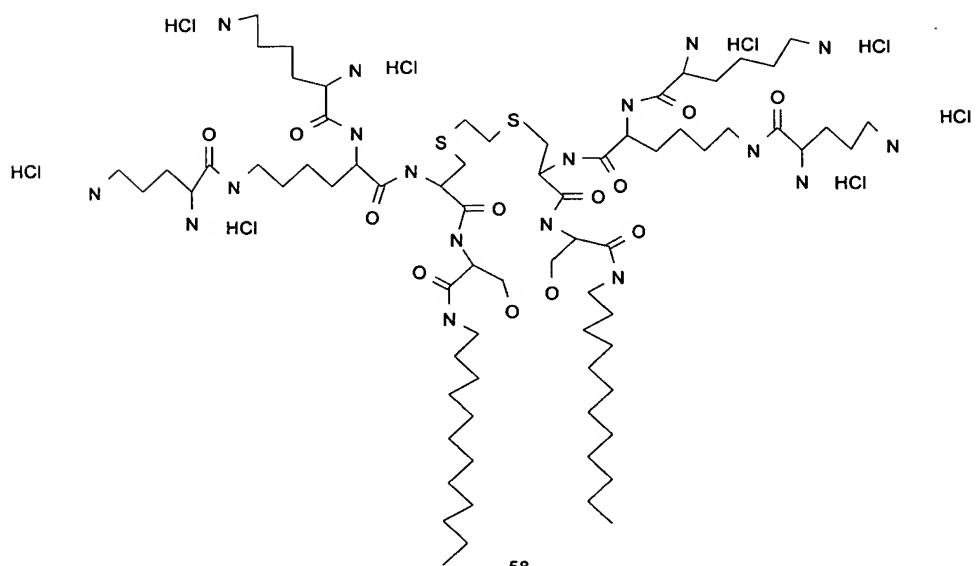
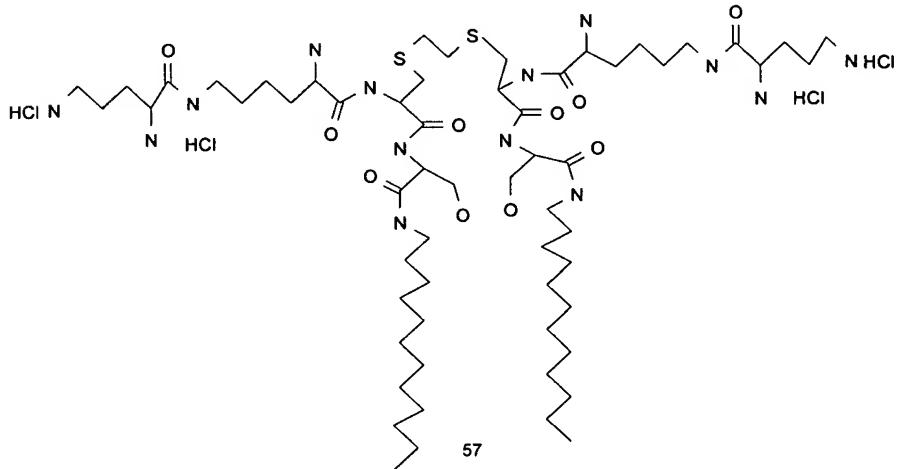
Example 15. Compound 56

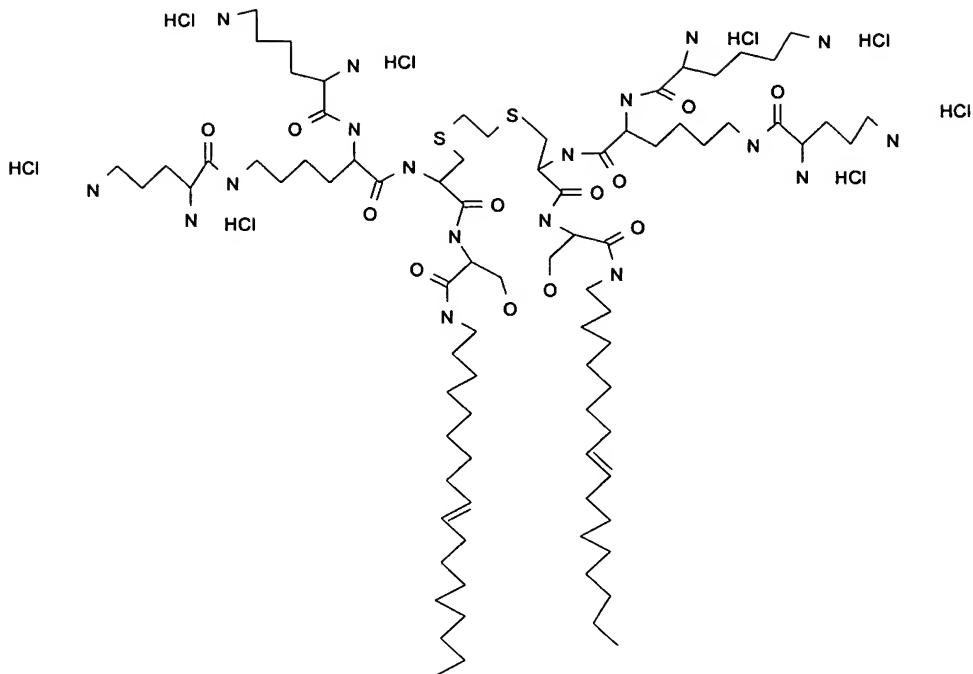
Compound 56 was synthesised as for compound 55 except that 1.95g (4.4mmole) 48 was used. This yielded 1.1g (60%) 56 as an off white solid.



Example 16. Compounds 57, 58 and 59

Compounds 57, 58 and 59 are synthesised in a similar manner to the compounds described above. Compound 39, or an intermediate equivalent to compound 39 but having different saturated or unsaturated hydrocarbon chains, is combined with an ornithine compound using synthetic peptide chemistry well known to the skilled person.





59

It will be appreciated by a person skilled in the art that in the formulae shown in the examples above, the hydrogen atoms have been omitted from the N, C and O atoms, where appropriate, for clarity.

Example 17. Transfection of recombinant plasmid expressing luciferase into HEK293 cells using peptide-based gemini compounds.

All tissue culture reagents were obtained from Life Technologies Inc. HEK 293 cells were seeded at $2-3 \times 10^5$ cells per well in Nunc six-well culture plates, 24 hours prior to transfection. The cells were seeded in 2mls Dulbeccos Modified Eagle medium containing Earles salts and supplemented with 10% v/v foetal bovine serum (= complete medium). The cells were grown at 37°C in 5% CO₂ in a humidified atmosphere. 6ug DNA ("luciferase control plasmid" from Promega Corp.) were dissolved in 100ul serum free medium (OPTI-MEM[®]). The peptide-based gemini compounds were made up at 1mg/ml in tissue culture grade water and then diluted in OPTI-MEM[®] to the appropriate concentration to a final volume of 100ul. The DNA and gemini solutions were mixed (to a total volume of 200ul; final concentrations of

5, 25, 50, 100, 150, 200, 250 and 300ug/ml) and left at room temperature for 15 minutes. The DNA/ gemini mix was placed onto the cells in each well and left in contact for 18-20 hours. The cells were then washed twice with phosphate buffered saline prior to 1ml of fresh complete medium being added. Cells were incubated for 5 a further 24 hours prior to lysis and luciferase activity assayed.

All luciferase activity assays were performed using the Canberra Packard (Berkshire, UK) Luclite kit according to the manufacturer's instructions with the exception that the cells in each well were resuspended in 1ml lysis buffer and 100ul aliquots mixed 10 with 100ul of the luciferase substrate. The reaction mix was left for a 15 minutes adaptation period in the dark before counting for 5 minutes in a Top Count scintillation counter. Luciferase activity is measured as counts per second (CPS) from the scintillation counter. Four independent counts were taken per well.

15 Control transfections were set up with no DNA, CaPO₄, an anionic gemini compound (1) and the commercially available lipofection reagents LipofectAmineTM and LipotaxiTM at the manufacturers recommended concentrations (10, 25, 50, 75, 125ug/ml and 175, 250, 325, 400 and 500ug/ml respectively).

20 The results (figure 1) clearly show that the cationic peptide-based gemini compounds (54), (55) and (56) are very efficient agents for facilitating the transfection of the luciferase plasmid into HEK293 cells at concentrations above 150ug/ml. In particular compound (54) peaks at 250ug/ml with a mean count (of 4 independent 25 counts) of over 70,000cps. Compound (55) is most effective at 300ug/ml with an average count of about 45,000cps. Compound (56) is most effective at 200ug/ml with an average count of about 50,000cps. In contrast the 'no DNA' negative control gives a background count as do the anionic gemini (1) and the cationic geminis (50 and 52). The CaPO₄ transfection shows a very low count of about 2,000cps. In 30 comparison figure 2 shows the results for the Lipofectamine transfections which at peak efficiency gave only 12,500cps (125ug/ml) and Lipotaxi 2,500cps (at 175ug/ml and 325ug/ml).

Example 18. Transfection of recombinant plasmid expressing luciferase into CHO-K1 cells using peptide-based gemini compounds.

CHO-K1 cells (ATCC: CRL-9618) were seeded into T₂₅-culture flasks (Corning-Costar Buckinghamshire, UK), at 7 x 10⁵ cells per flask, 24 hours prior to

5 transfection. The CHO-K1 cells were seeded in 5 ml MEM alpha medium with ribonucleosides and deoxyribonucleosides and supplemented with 1x L-glutamine and 10 % v/v foetal bovine serum (complete medium). The cells were grown at 37°C in 5 % CO₂ in a humidified atmosphere.

For transfection, 5 ug DNA (luciferase control plasmid) was incubated with the 10 gemini compounds in water (final volume 400 µl). The peptide-based gemini compounds were made up at 1 mg ml⁻¹ in tissue culture grade water and then diluted to the appropriate concentration to a total volume of 200 µl. Following a 30 minute room temperature incubation, 2.6 ml OPTI-MEM® medium was added and the solution placed on the cells. Following an overnight incubation at 37°C, the 15 transfection solution was replaced with complete medium and the cells incubated at 37°C. 24 hours post transfection the cells were detached from the flask and seeded into 96-well plates at a density of 0.5 x 10⁵ cells per well and incubated for a further 24 hours at 37°C. Luciferase reporter gene assays were performed according to the manufacturers instructions (Roche Diagnostics, Mannheim, Germany) approximately 20 48 hours post transfection. The plates were left for a 15 minutes adaption period in the dark before counting for 60 seconds in a TopCount NXT counter (Canberra Packard). An average of eight wells were counted per transfection.

Control transfections were set up with no DNA, an anionic gemini compound and the commercially available reagent LipofectAmine PLUS™.

25 The results, shown in figure 3, demonstrate that the cationic peptide-based compounds 54, 55, and 56 are very efficient agents for facilitating the transfection of the luciferase plasmid into CHO-K1 cells. Using the conditions described above, compound 54 peaks at 30 mM with a mean count in excess of 1.4 x 10⁵ counts per 30 second (cps). Compound 55 is most effective at 30 mM with an average count of about 2.4 x 10⁵ cps. Compound 56 is most effective at 40 mM with an average count of about 1.9 x 10⁵ cps. In contrast negative controls gave a negligible count.

Example 19. Transfection of recombinant plasmid expressing luciferase into CHO-K1 cells using peptide-based gemini compounds in combination with various supplements.

The transfection ability of the gemini compounds could be further enhanced by the 5 addition of a neutral carrier, for example, dioleyl phosphatidylethanolamine (DOPE) (Farhood, H., *et al* (1985) *Biochim. Biophys. Acta* 1235 289) or a complexing reagent, for example, PLUS compound (Life Technologies Inc.).

Figure 4 shows, for example, a 9-fold increase of luciferase activity at a 2:1 ratio of compound 55 and DOPE. Transfection mediated by compound 55 with DOPE in a 10 2:1 ratio and the addition of 11.6 ul of PLUS compound lead to a mean count of 6.5×10^5 cps representing a 12-fold increase of luciferase activity in comparison to compound 55 alone. Incubation of the PLUS compound with the DNA and combination with compound 55 alone also lead to a 4-fold increase.

15 **Example 20. Use of peptide-based gemini compounds to facilitate adhesion of cells in culture to the culture flask.**

Using normal growth medium and culture conditions (RPMI plus 10% foetal bovine serum; 37°C, 5% CO₂) but with the addition of 50-60ug peptide-based gemini compound per well, it was observed that with the suspension cell line Jurkat, cells 20 could attach to the bottom surface of the plastic culture vessel. In the absence of gemini compounds, the Jurkat cells grew in suspension.